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Cross Protection Study in Feline Hemoplasmas

Inaugural-Dissertation

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Summary

‘*Candidatus* Mycoplasma turicensis’ (CMt) and ‘*Mycoplasma haemofelis*‘ (Mhf) are feline hemoplasmas that may induce anemia. Cats that overcome CMt infection demonstrate high antibody levels and are protected from a subsequent CMt challenge. In this study we aimed to investigate a possible cross protection between CMt and Mhf and characterize low-dose subcutaneous Mhf infection. Ten specified pathogen-free cats were exposed subcutaneously to 1’000 copies of Mhf: five cats were chronically infected with CMt (PCR-negative in blood but serologically positive, group A) and five cats served as naïve controls (group B). Bacterial loads were detected by real-time PCR. Hematology, clinical chemistry and serology were monitored. All ten cats became Mhf PCR-positive; cats in group A became significantly earlier Mhf PCR-positive and anemic and showed significant higher antibody levels than cats in group B. Mhf was demonstrated in saliva, rectal swabs and urine for the first time, although at low copy numbers. In conclusion, in contrast to the CMt-CMt-reinfection experiment, cats that recovered from CMt infection were not protected from a subsequent Mhf challenge. Our results may partly explain the diverse outcomes of Mhf infections observed in individual domestic cats. Furthermore, a small volume of Mhf infectious blood was sufficient for the induction of hemoplasmosis. We propose to use the low-dose challenge in future hemoplasma studies, which may more accurately mirror the natural way of transmission.

Zusammenfassung

‘*Candidatus* Mycoplasma turicensis’ (CMt) und ‘*Mycoplasma haemofelis*‘ (Mhf) sind feline Hemoplasmen, die eine Anämie auslösen können. Katzen, die eine CMt-Infektion überstanden haben, weisen hohe Antikörpertiter auf, welche eine schützende Wirkung auf eine darauffolgende CMt-Infektion haben. Das Ziel unserer Studie war es, eine potentielle Kreuzimmunität zwischen CMt und Mhf aufzuzeigen, sowie die Charakterisierung der Infektion mit einem niedrig dosierten Mhf Inokulum. Zehn spezifisch pathogenfreie Katzen wurden subkutan mit 1'000 Mhf Kopien infiziert: Fünf der Katzen waren chronisch mit CMt infiziert (PCR-negativ, serologisch positiv, Gruppe A) und fünf naive Katzen dienten als Kontrolle (Gruppe B). Hämatologie, klinische Chemie, Serologie und real-time PCR dienten der Überwachung der Infektion. Katzen der Gruppe A wurden sowohl signifikant früher PCR-positiv, als auch anämisch und zeigten höhere Antikörpertiter als Katzen der Gruppe B. Zum ersten Mal konnte Mhf in Speichel- und Kottupfern, sowie im Urin nachgewiesen werden. Schlussfolgernd konnte aufgezeigt werden, dass Katzen, die sich von einer CMt-Infektion erholt haben, nicht vor einer darauffolgenden Mhf-Infektion geschützt sind. Desweiteren wurde festgestellt, dass eine Hämoplasmose bereits durch ein minimales Volumen von Mhf infiziertem Blut ausgelöst werden kann. Für zukünftige Hämoplasmen-Studien empfehlen wir deshalb die Benützung der niedrig dosierten Mhf-Inokula, um einen möglichst naturnahen Weg der Übertragung zu imitieren.

1. Introduction

Hemotropic mycoplasmas (hemoplasmas) are cell-wall free bacteria, which can attach to red blood cells and may induce hemolytic anemia. Various mammalian species, including pets, as well as wild animals, can be infected with the bacteria. In the domestic cat three feline hemotropic mycoplasma species are known: '*Mycoplasma haemofelis*' (Mhf), '*Candidatus* Mycoplasma haemominutum' (CMhm) and '*Candidatus* Mycoplasma turicensis' (CMt) [1-4]. Feline hemotropic mycoplasmas have been demonstrated worldwide with different prevalences [5-8]. The pathogenic potential significantly varies among the three feline species; Mhf seems to be the most pathogenic of the three species and an acute infection often results in hemolytic anemia [9, 10]. Clinical signs of a hemoplasmosis may include pallor, lethargy, anorexia, depression, dehydration and fever [10]. CMt was first isolated in 2005 in a Swiss pet cat [1]. Infection with CMt may result in a reduction of red blood cells (RBC) and hematocrit, but frequently no severe anemia was observed in CMt-infected cats. CMt-positive cats co-infected with CMhm or Mhf, in contrast, may show severe anemia [6].

The route of transmission of feline hemoplasmas is still unknown, but arthropods such as fleas or ticks are suspected to play an important role [11-14]. Aggressive interactions between cats as well as blood transfusions have also been associated with transmission of hemotropic mycoplasma [15-17]. In experimental studies, the intraperitoneal and intravenous inoculation of large volumes of infectious blood was used to induce infection [9, 18-20]. However, these two routes of transmission seem to be a rather inadequate model for natural transmission. Results from a previous study with CMt [17] suggested that the subcutaneous inoculation and the use of small volumes of infectious blood, would mimic a more natural way of transmission of feline hemotropic mycoplasmas. So far, no data is available on low-dose Mhf infection.

The diagnosis of feline hemotropic mycoplasma infections relies on the detection and differentiation of the infectious agents by sensitive Polymerase Chain Reaction (PCR) assays [1, 21]. Furthermore, hemotropic mycoplasmas can be identified on stained blood smears, especially during high bacteremia. Hemoplasmas appear as small epicellular coccoid structures on the surface of the red blood cells [22]. To quantify the humoral immune response to feline hemoplasmas an enzyme-linked immunosorbent assay (ELISA) based on a recombinant Mhf DnaK protein was used [18, 23].

The pathogenesis of hemotropic mycoplasma infections in cats is still poorly understood. Recently, it has been discovered, that cats previously exposed to CMt demonstrated high levels of antibodies even months after the initial bacteremia and that these cats were protected from a subsequent CMt challenge [24].

The aims of the present study were to investigate subcutaneous low-dose Mhf infection and characterize the course of infection and the shedding pattern of Mhf. Furthermore we aimed to investigate a possible cross protection between CMt and the more pathogenic Mhf.

2. Materials and methods

2.1. Animals and experimental design

Ten adult male SPF cats (FIA1, FIA2, FHT1, FHX4, FHX5, KCY2, ZKA2, AKL4, JCT2, KCU1) were included in the present investigation. The cats were divided in two groups (groups A and B). Group A consisted of five cats (FIA1, FIA2, FHT1, FHX4, FHX5), which had previously undergone CMt infection [25]. After the cats had overcome the acute infection, they were PCR-negative for CMt, but serologically positive. All cats in group A were five years old at the time point of the start of the study. Group B consisted of five naïve cats (KCY2, ZKA2, AKL4, JCT2, KCU1). Three of the cats in group B (KCY2, JCT2, KCU1) were three years old; two cats (ZKA2, AKL4) were six years old at the beginning of the experiment. The ten cats were of blood type A.

All animal experiments were performed according to the Swiss law and were officially approved by the veterinary office of the canton Zurich (TVB 159/2010). The cats were kept in two groups under etiologically and hygienically ideal conditions as described [26] and prior to the start of the study, the SPF status of the cats was verified, as described previously [17].

All cats were challenged subcutaneously at day 0 with 1’000 copies of Mhf as determined by 16S rRNA TaqMan® real-time PCR. The way of inoculation and the dose of Mhf were chosen according to previous successful experimental CMt infections [17]. Infectious blood was diluted in a final volume of 100 µL (see below). EDTA-anticoagulated blood and serum samples were collected for PCR analysis, hematology, clinical chemistry and serology from the ten cats before and regularly after the Mhf inoculation (Figures 1 and 2). Saliva and rectal swabs were collected regularly (time

points indicated in Figure 1) using commercially available cotton swabs (Primella, Migros Genossenschafts-Bund, Zurich, Switzerland) as described [13]. To look at the long-term shedding, we additionally tested the saliva and rectal swabs collected at days 141, 232 and 286 post infection. Moreover, urine samples of all cats were collected by cystocentesis at three time points, one before and two after the Mhf inoculation (days -1, 57 and 104 post Mhf exposure). At the three time points, urinalysis was performed and the urine sediment was evaluated. Clinical condition, body temperature and body weight were recorded at each sampling time point. Cats were monitored for 371 days post Mhf exposure. Mhf-infected cats, which developed severe anemia (PCV < 10%) and/or were in a poor general condition were treated with doxycycline orally (10 mg/kg/d, Grünenthal GmbH, Mitlödi, Switzerland) for 14 days, prednisolone orally (2 mg/kg every 12h, gradually withdrawn, Streuli Pharma AG, Uznach, Switzerland) for ten days and fluid therapy (Ringer's lactate solution, Fresenius Kabi (Schweiz) AG, Stans, Switzerland).

2.2. Preparation of inoculum

Heparinized infectious blood from cat QLA5 [18] preserved in dimethylsulfoxide (20%, vol/vol) in liquid nitrogen was used to prepare the inocula for the ten experimental cats. The blood contained 2.2 x 10^7 copies of Mhf/mL as determined by 16s rRNA TaqMan® real-time PCR. It was thawed at 37ºC and diluted in cold phosphate-buffered saline to a final concentration of 1 x 10^3 copies in 100 µL. The inoculum was kept on ice until use. Each cat was injected within five minutes of the preparation of the inoculum subcutaneously with 100 µL in the region of the neck.

2.3. Hematology and Coombs‘ test

White blood cell differentials and complete hemograms were performed using a Sysmex XT-2000iV (Sysmex Corporation, Kobe, Japan) [27]. Packed Cell Volume (PCV) values between 33% and 45% were considered to be within the reference range as determined in our laboratory by using identical methods and blood samples from 63 clinically healthy cats; anemia was defined as a PCV value below 33%. In addition blood smears were prepared manually and were Giemsa stained using a Hema Tek 1000 (Bayer AG, Zurich, Switzerland). Blood smears were evaluated by light microscopy for Mhf.

After the observation period of this study (371 days), the cats stayed in the facility under unchanged conditions. Coombs' test was performed at different time points after the end of the observation time (at days 364, 387, 390 and 427 - 429 post Mhf exposure). Coombs' testing was done as described previously [1], with the following modification: the feline anti-globulin Coombs' reagent (ImmunO™, MP Biomedicals, Solon, Ohio) was serially diluted from 1:2 to 1:10'240.

2.4. Clinical chemistry

Serum chemistry was performed using a Cobas Integra 700 system (Roche Diagnostics, Rotkreuz, Switzerland) and included bilirubin, glucose, blood urea nitrogen (BUN), creatinine, protein, albumin, cholesterol, triglyceride, alkaline phosphatase, amylase, aspartate aminotransferase, alanine aminotransferase, lipase, sodium, chloride, potassium, calcium and phosphate. Reference ranges are stated as 5% and 95% quantiles; they were determined in our laboratory using identical methods and blood samples from 59 clinically healthy cats.

2.5. Serum protein electrophoresis

Serum protein electrophoresis was performed at selected time points before and after the Mhf infection (days -1, 44, 56, 58 and 69 post Mhf exposure). A semi-automated agarose gel electrophoresis system (Hydragel-Hydrasis, Sebia PN 4100, Issy-les Moulineaux, France) was used according to the procedure described by the manufacturer (Hydragel 7 Protein kit, Sebia PN 4100). Control serum (control serum P human, Sebia, Lysses, France) was included in each run. The relative protein concentration of each fraction was determined as the percentage optical absorbance, and the absolute concentration of each fraction (g/L) was calculated by multiplying the percentage with the total serum protein concentration. The reference ranges were determined in our laboratory using identical methods and serum samples from 59 clinically healthy cats and are stated as 5% and 95% quantiles.

2.6. Nucleic acids extractions

Total nucleic acids (TNA) were extracted from 100 µL EDTA-anticoagulated blood using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostic, Rotkreuz, Switzerland), as described previously [1]. The saliva and rectal swabs were incubated in phosphate-buffered saline at 40ºC for ten min prior to TNA extraction, as previously described [28]. TNA of urine samples were extracted from 100 µL urine using the MagNA Pure LC DNA Isolation Kit III (Bacteria, Fungi) (Roche Diagnostic, Rotkreuz, Switzerland). As pre-isolation steps, bacterial lysis buffer and proteinase K were added and the mixture was incubated at 65ºC for ten min. The subsequent procedure was according to the manufacturer's instructions.

TNA was eluted into 100 µL elution buffer and stored at -20ºC until PCR testing was performed. During all extractions, negative controls consisting of 100 µL phosphate-buffered saline were concurrently prepared with each batch of samples to monitor for cross-contamination.

2.7. Quantitative TaqMan® real-time PCR assays

All TNA samples were tested by TaqMan® real-time PCR for the presence and load of Mhf and CMt on an ABI PRISM 7700/7500 Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland), as previously described [1, 16]. All PCR runs were performed with positive and negative controls.

2.8. Serology

An ELISA based on a recombinant Mhf DnaK protein [18] was used in order to quantify the magnitude of the humoral immune response after Mhf exposure in the naïve cats and in the cats with preexisting anti-CMt-antibodies. The DnaK-ELISA was modified using a serum dilution of 1:500. The signal-to-noise ratio was calculated dividing the post- by the pre-infection absorbance values for each individual cat as described [18]. An ELISA signal ratio of ≥ 1.5 was considered serologically positive. Samples collected up to 371 days post Mhf exposure were included in the measurements.

2.9. Statistics

Statistical analyses were performed using Graph-Pad Prism Version 3.0 (GraphPad Software, San Diego, CA, USA) and nonparametric tests. The parameters were compared between two groups using the Mann-Whitney U-test (P_{MWU}). The Wilcoxon signed-rank test (P_W) was used to compare the serum protein electrophoresis results from the same cat at two different time points. The Friedman's test (P_F), followed by the Dunn's post test was used to analyze the parameters over time when more than two time points were considered. For the correlation analyses, the Spearman rank correlation test was used (P_S). P values < 0.05 were considered to be significant.

3. Results

3.1. Outcome of Mhf challenge and Mhf blood loads

At the start of the study, the blood of all ten cats was Mhf- and CMt-negative as determined by TaqMan® real-time PCR. All cats turned Mhf PCR-positive during the experiment. CMt was never detectable by PCR in the blood of any of the cats. Among the cats in group A, cat FIA1 became Mhf PCR-positive at day nine, cats FHX4 and FHX5 at day 13 after the exposure and finally cats FIA2 and FHT1 became Mhf PCR-positive at day 16 post exposure (Figure 1). KCY2 and AKL4 became PCR-positive at days 20 and 23 after the Mhf exposure, respectively; cats ZKA2 and KCU1 turned positive 27 and 30 days post exposure. The last cat to become PCR-positive was JCT2 at day 34 after subcutaneous inoculation of Mhf. This was the only cat which showed some PCR-negative results unrelated to the antibiotic treatment (at days 37 and 41) within the first weeks after the cat had become PCR-positive. Moreover, this cat demonstrated the most severe outcome of the infection. Overall, cats in group A became Mhf PCR-positive within a range of 9 to 16 days post exposure, while cats in group B turned PCR-positive significantly later (in a range of 20 to 34 days post exposure, P_{MWU} = 0.008). The maximum Mhf blood loads were comparable in both groups and reached ~10^9 copies/mL blood in each of the ten cats. Some marked copy number fluctuations were seen in the course of the Mhf infection in all cats, but particularly in a cat in group B, cat ZKA2.

Figure 1: Outcome of experimental subcutaneous Mhf exposure (at day 0) in ten SPF cats: five CMt-chronically infected cats (A-E), five naïve cats (F-J). Cat FIA1 (A), cat FIA2 (B), cat FHT1 (C), cat FHX4 (D), cat FHX5 (E), cat KCY2 (F), cat ZKA2 (G), cat AKL4 (H), cat JCT2 (I) and cat KCU1 (J). The blood loads are presented as log DNA copy numbers per mL of blood (left y-axis) as determined by 16S rRNA TaqMan® real-time PCR and the PCV values are given as a percentage (right y-axis). The PCR results from saliva and rectal swabs are illustrated as triangles below the x-axes. PCR-positive swabs are demonstrated as filled symbols; PCR-negative swabs are shown by open symbols. Only the first 90 days PI are shown. Cat JCT2 was treated as indicated with doxycycline (gray box marked “D”) and prednisolone (white box marked “P”).

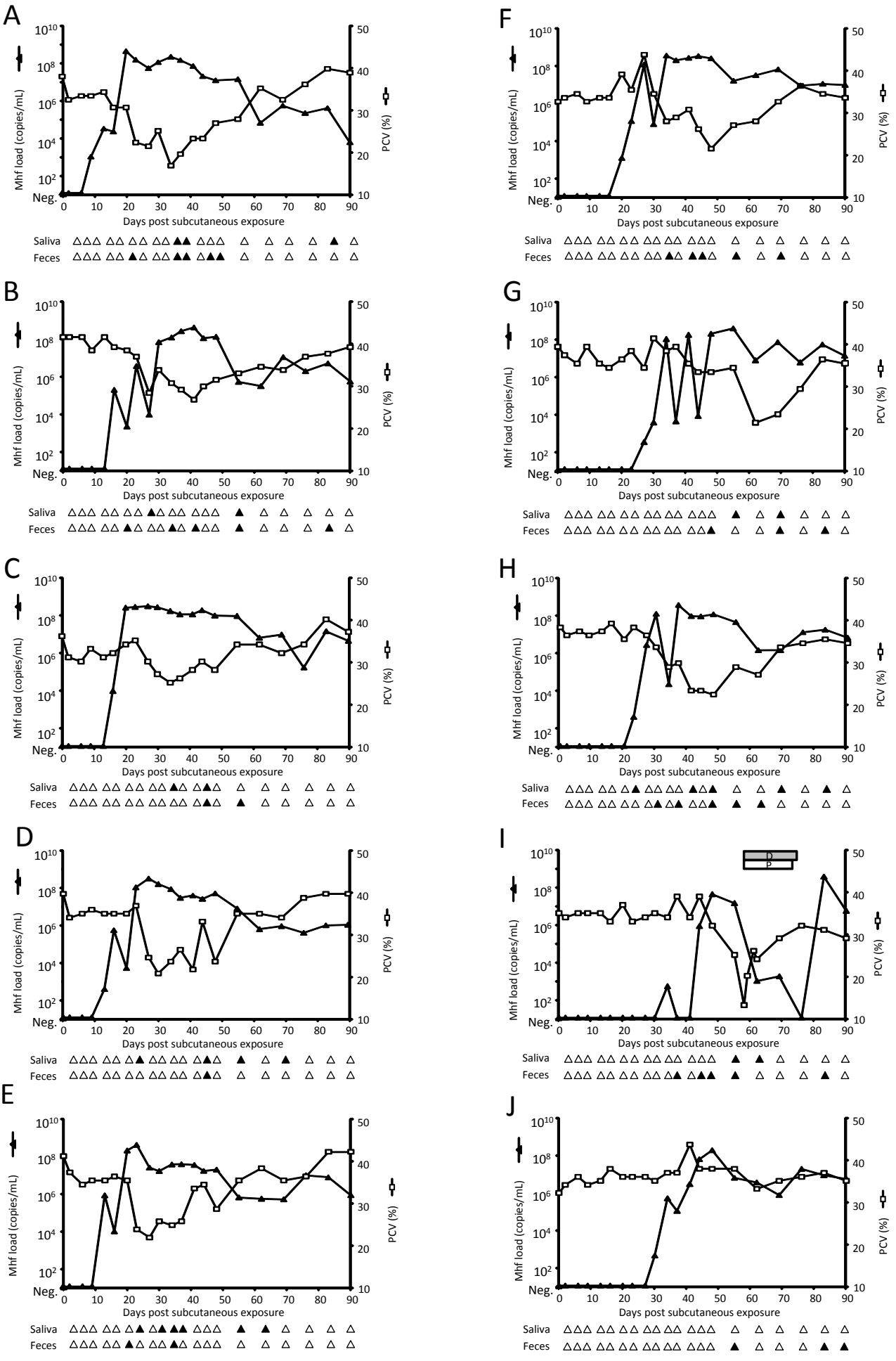
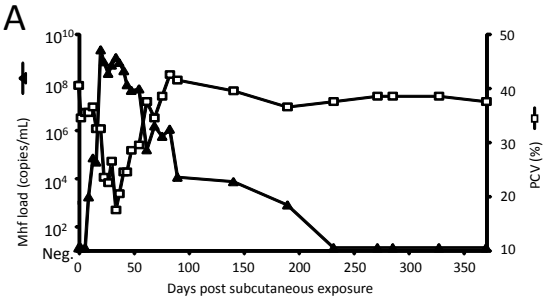
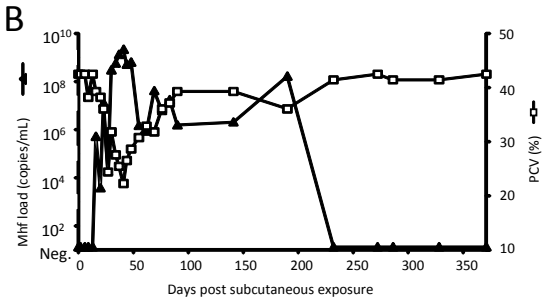
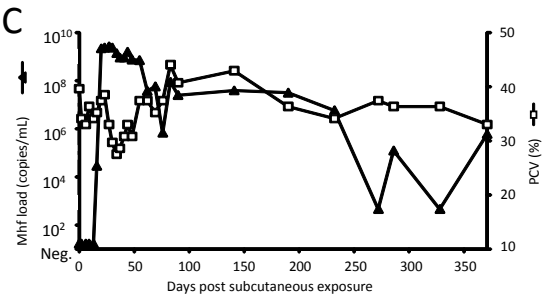
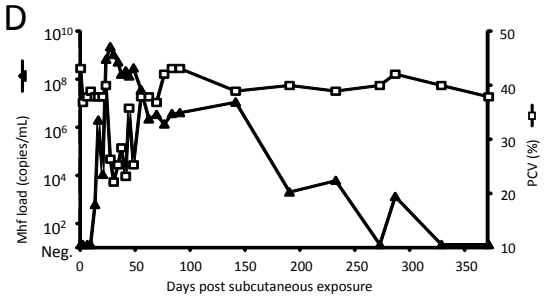

A
B
C
D
E
F
G
H
I
J
Mhf load (copies/mL)
PCV (%)
Days post subcutaneous exposure
Saliva
Feces
Neg.

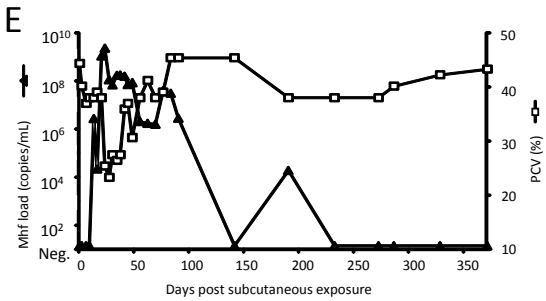
Figure 2: Long-term follow-up of experimental subcutaneous Mhf exposure (at day 0) in ten SPF cats: five CMt-chronically infected cats (A-E), five naïve cats (F-J). For detailed cat identifications refer to Figure 1. The blood loads are presented as log DNA copy numbers per mL of blood (left y-axis) and the PCV values are given as percentage (right y-axis).

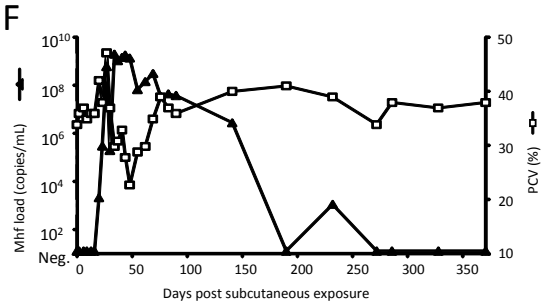

A
Mhf load (copies/mL)
PCV (%)
Days post subcutaneous exposure

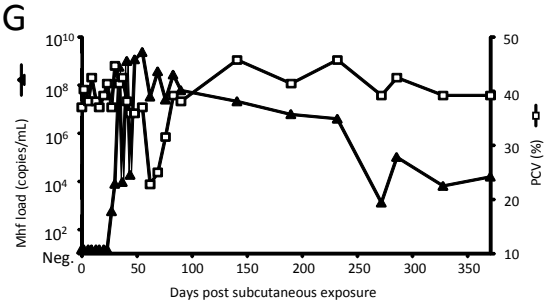

B
Mhf load (copies/mL)
PCV (%)
Days post subcutaneous exposure

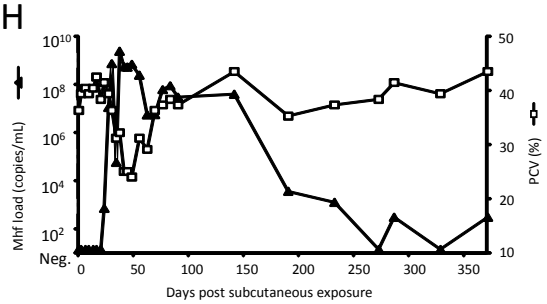

C
Mhf load (copies/mL)
PCV (%)
Days post subcutaneous exposure

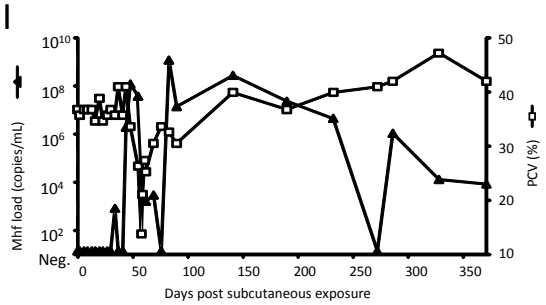

D
Mhf load (copies/mL)
PCV (%)
Days post subcutaneous exposure


E
Mhf load (copies/mL)
PCV (%)
Days post subcutaneous exposure


F
Mhf load (copies/mL)
PCV (%)
Days post subcutaneous exposure


G
Mhf load (copies/mL)
PCV (%)
Days post subcutaneous exposure


H
Mhf load (copies/mL)
PCV (%)
Days post subcutaneous exposure


I
Mhf load (copies/mL)
PCV (%)
Days post subcutaneous exposure


J
Mhf load (copies/mL)
PCV (%)
Days post subcutaneous exposure

3.2. Clinical outcome

Two cats in group A (FIA1, FIA2) and three cats in group B (KCY2, ZKA2, JCT2) showed transient clinical signs of hemoplasmosis, within the first nine weeks after subcutaneous inoculation of Mhf. Cats FIA1, FIA2, KCY2, ZKA2 were depressed, had pale mucous membranes and showed a reduced appetite over a period of a few days. No significant changes in body temperature or body weight were observed in any of the cats.

At day 58 post Mhf exposure, cat JCT2 (group B) showed severe clinical signs like an enlarged abdomen (non-painful), pale mucous membranes, apathy, anorexia and an increased salivation. After evaluating the hematological parameters (PCV of 13%), the cat was treated with doxycycline and prednisolone as described in materials and methods. One week after the end of the doxycycline therapy, the cat turned PCR-negative (Figure 1). In the subsequent week the cat became PCR-positive again; it showed a marked increase in Mhf copy numbers and reached 10^8 copies/mL of blood, the highest load measured in this cat.

3.3. Hematology and Coombs' test

All cats in group A and four out of five cats in group B developed anemia (PCV < 33%) throughout the observation time (Figure 1). The cats in group A showed the onset of anemia 16 to 27 days post Mhf exposure and 7 to 14 days after the animals had turned PCR-positive. The PCV and RBC counts in cats in group A transiently decreased significantly at day 34, which corresponds to 18 to 25 days after the cats had turned PCR-positive (PCV $P_F < 0.0001$; RBC < 7 x 10^6/µL, $P_F < 0.0001$). In the four naïve cats in group B, which developed anemia, the onset of anemia was significantly later (34 to 62 days post Mhf exposure and 11 to 35 days after becoming PCR-positive, P_{MWU} =

0.03) than in the cats in group A. The PCV and RBC values in cats in group B transiently decreased at day 62, which relates to 28 to 42 days after the onset of PCR-positivity (RBC P_F = 0.04). The lowest PCV value was measured in cat JCT2 (group B) at day 58 post exposure (PCV = 13%).

One month after the cats turned PCR-positive, a significant increase in monocytes (P_F < 0.0001) and a significant decrease in eosinophiles (P_F < 0.0001) could be observed (Figure 3). No significant changes were found in total leucocyte, neutrophil and lymphocyte counts, when the hematological parameters were followed over time. In addition, no significant differences were found for total leucocyte and differential counts. Neutropenia (< 2.3 x 10^3/µL) was observed in several cats in both groups throughout the experiment, but particularly in cat JCT2 (group B). This cat showed severe neutropenia at different time points throughout the observation time. None of the cats showed any positive results in the Coombs' test at the selected time points tested after the Mhf exposure.

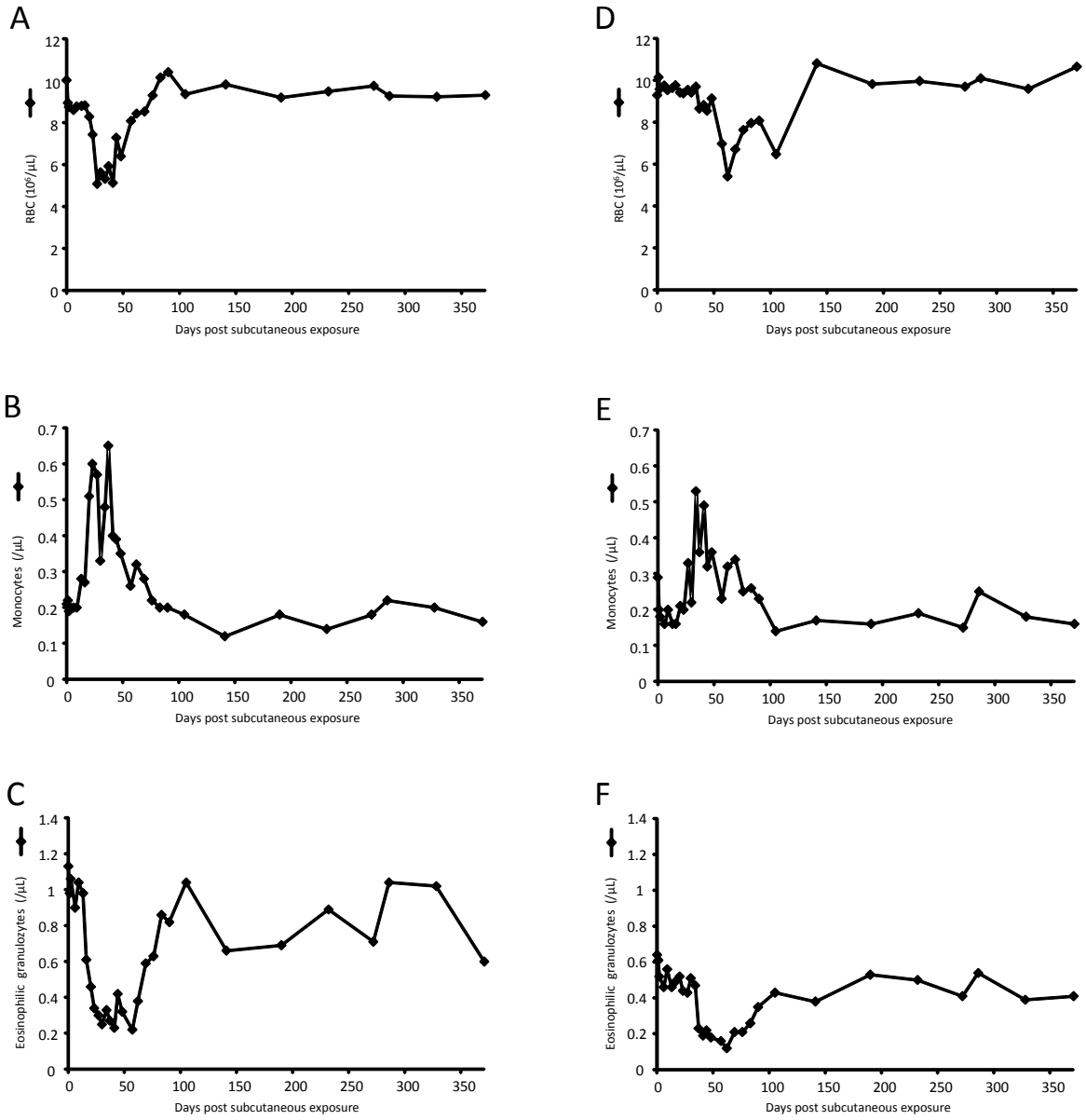


Figure 3: Outcome of selected hematological parameters after experimental subcutaneous Mhf exposure (at day 0) in ten SPF cats: five CMt-chronically infected cats (A-C), five naïve cats (D-F). Median RBC (A, D), monocytes (B, E) and eosinophilic granulocytes (C, F) concentrations (y-axis) plotted against days post subcutaneous Mhf exposure.

3.4. Blood smears

A selected sample of modified Wright stained blood smears, at time points during high bacteremia, was screened by light microscopy. Small epicellular coccoid structures could be identified on the surface of the red blood cells, they were found either individually, in pairs or as lined chains (Figure 4). Furthermore, anisocytosis, polychromatic erythrocytes and normoblasts were seen.

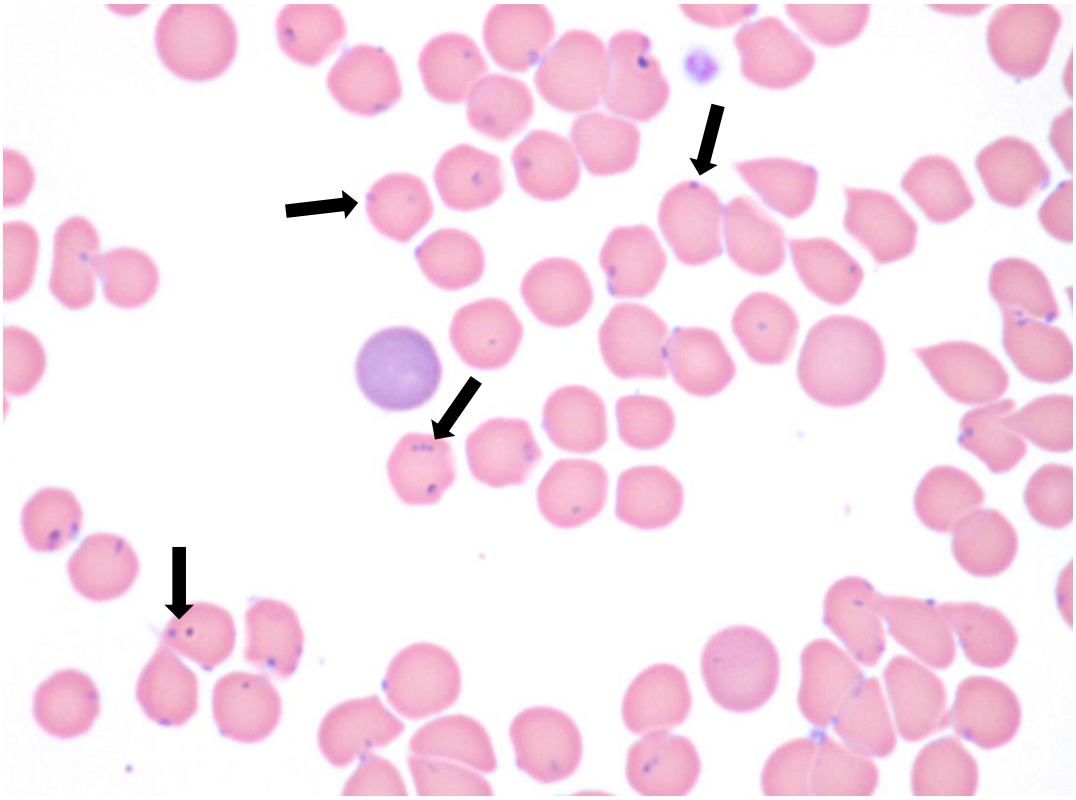


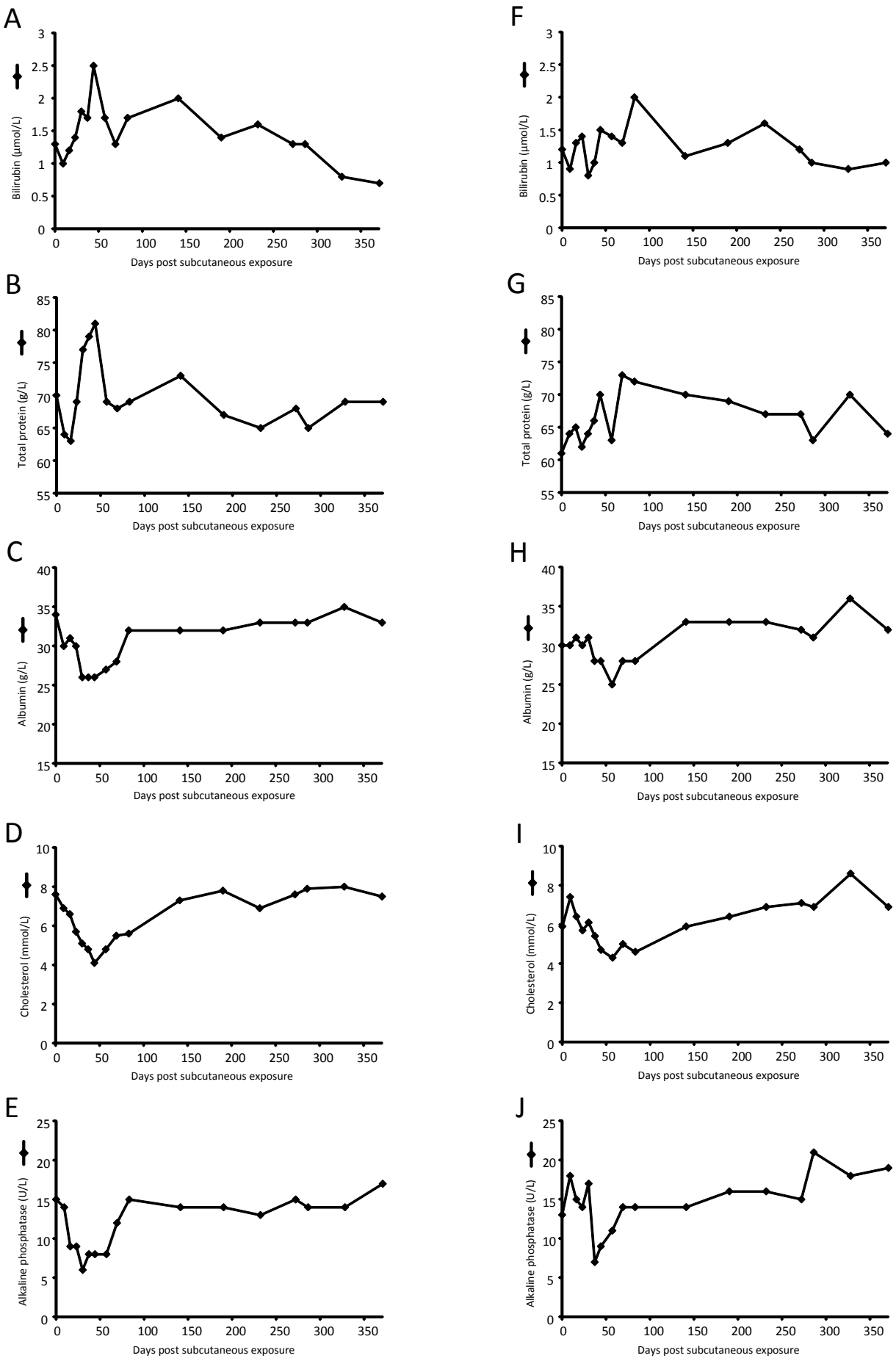
Figure 4: Modified Wright stained blood smear from cat AKL4 (group B), 38 days after low-dose Mhf exposure. Small epicellular coccoid structures could be identified on the surface of the red blood cells (black arrows). Anisocytosis and polychromatic erythrocytes could also be identified compatible with regenerative anemia.

3.5. Clinical chemistry

The cats showed significant changes in different clinical chemistry parameters during the observation time. One month after the cats in group A became PCR-positive, a significant increase in bilirubin ($P_F < 0.05$) and total protein concentration ($P < 0.01$), as well as a significant decrease in albumin ($P_F < 0.001$), cholesterol ($P_F < 0.01$) and alkaline phosphatase ($P_F < 0.001$) concentration could be observed (Figure 5).

Cats in group B presented similar tendencies but not as pronounced as cats in group A. One month after becoming PCR-positive, the cats in group B had a significant increase in total protein concentration ($P_F < 0.01$) and showed a significant decrease in albumin ($P_F < 0.001$) and cholesterol ($P_F < 0.001$). Glucose, blood urea nitrogen (BUN), creatinine, triglyceride, amylase, aspartate aminotransferase, alanine aminotransferase, lipase, sodium, chloride, potassium, calcium and phosphate blood levels did not show any significant changes. One cat in group B (JCT2) showed an increase in aspartate aminotransferase (108 U/l) and alanine aminotransferase (806 U/l) above the reference range at day 69 post exposure.

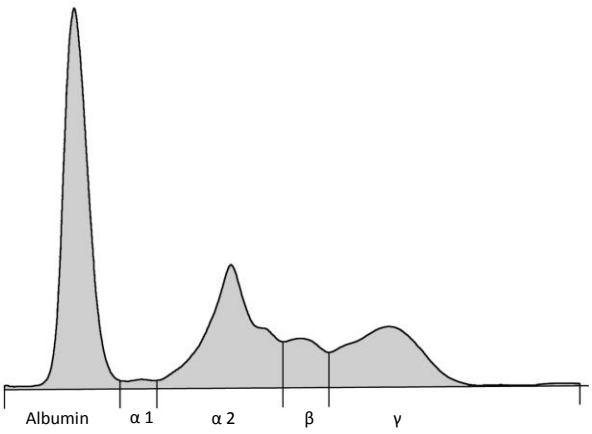
Figure 5: Long-term follow-up of selected clinical chemistry parameters after experimental subcutaneous Mhf exposure (at day 0) in five CMt-chronically infected cats (A-E) and five naïve cats (F-J). Median bilirubin (A, F), total protein (B, G), albumin (C, H), cholesterol (D, I) and alkaline phosphatase (E, J) concentration (y-axis) plotted against days post subcutaneous Mhf exposure.


A
Bilirubin (µmol/L)
Days post subcutaneous exposure
B
Total protein (g/L)
Days post subcutaneous exposure
C
Albumin (g/L)
Days post subcutaneous exposure
D
Cholesterol (mmol/L)
Days post subcutaneous exposure
E
Alkaline phosphatase (U/L)
Days post subcutaneous exposure
F
Bilirubin (µmol/L)
Days post subcutaneous exposure
G
Total protein (g/L)
Days post subcutaneous exposure
H
Albumin (g/L)
Days post subcutaneous exposure
I
Cholesterol (mmol/L)
Days post subcutaneous exposure
J
Alkaline phosphatase (U/L)
Days post subcutaneous exposure

3.6. Serum protein electrophoresis

Different changes in serum protein electrophoresis could be observed at selected time points (Figure 6). A significant decrease in albumin concentration (P_W = 0.002) could be observed in all cats at day 58 after the Mhf exposure. Nine out of ten cats showed at least once during the observation time an albumin concentration below the reference range (<30-40 g/L). Simultaneously to this decrease in albumin, a significant increase in gamma globulines (P_W = 0.002) could be found in all cats. Nine of the cats had gamma globuline concentrations above the reference range (up to 40.2 g/L; reference range 5.7-16.0 g/L).

A



B

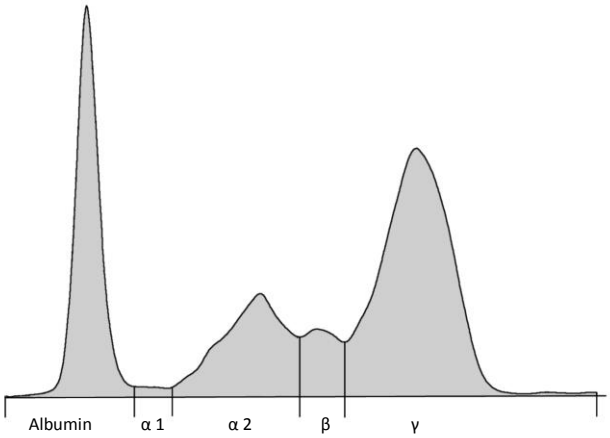


Figure 6: Representative serum protein electrophoresis of a cat (FIA1, group A) before the Mhf infection (A) and at day 44 post subcutaneous Mhf exposure (B). The electrophoretogram illustrates a polyclonal gammopathy at day 44 with a γ-globulin concentration of 40.2 g/L (reference range: 5.7 - 16.0 g/L).

3.7. Mhf DNA shedding via saliva, feces and urine

Saliva and rectal swabs were collected at the time points indicated in Figure 1 and at days 141, 232 and 286 post Mhf exposure. In all cats, some rectal swabs tested Mhf PCR-positive, while in only eight out of ten cats some saliva swabs were found positive. Two cats in group B (KCY2, KCU1) showed no PCR-positive saliva swabs during the whole observation time (Figure 1). The Mhf loads in saliva and feces were between 1 to 26 copies/reaction, which calculated to 200 to 780 copies/swab. Most of the positive results were found during the time of high bacteremia. All saliva and rectal swabs collected at days 141, 232 and 286 post Mhf exposure tested Mhf PCR-negative.

Urine samples were collected at three time points, pre- and post-infection. Mhf PCR-positive results were found in eight out of ten cats post Mhf exposure. The Mhf loads in urine were between 1 to 699 copies/reaction, which calculated to 2×10^2 to 1.4×10^5 copies/mL of urine. The highest Mhf level in urine was found in a sample with high erythrocyte contamination in urinalysis.

3.8. Humoral immune response to Mhf

The five naïve cats in group B were seronegative at the start of the study as determined by Mhf rDnaK ELISA (ELISA signal-to-noise ratio < 1.5; Figure 7). In these cats, the seroconversion took place seven to ten days after the cats became PCR-positive in the blood, except for cat JCT2, which seroconverted 21 days after PCR-positivity in blood (Figure 7B). The signal-to-noise ratio in this group reached a maximum of 17.6. The antibody levels increased after the peak Mhf blood loads and remained at a high level during the whole observation period for two cats (ZKA2, KCU1). For the three other cats in this group (KCY2, AKL4, JCT2) the level of antibodies decreased after reaching a peak (Figure 8B). The chronically infected cats (group A) were serologically positive (ELISA signal-to-noise ratio ≥ 1.5) at the beginning of this study (Figure 7A). In group A

an increase in antibody levels was observed three to ten days after the cats had become PCR-positive in blood. The signal-to-noise ratio in cats in group A reached a maximum of 30.3, which is significantly higher than the maximum in group B (P_{MWU} = 0.0079). Regarding the first 371 days post infection, the antibody levels were significantly correlated with the Mhf blood load (Spearmen correlation coefficient, r = 0.41; $P_S < 0.0001$).

3.9. Long-term outcome of the infection

Remarkably, five cats became PCR-negative in the blood for Mhf within the observation period without antibiotic treatment: four cats in group A and one cat in group B (Figure 2). The remaining six cats were still PCR-positive at the end of this project, 371 days after Mhf exposure.

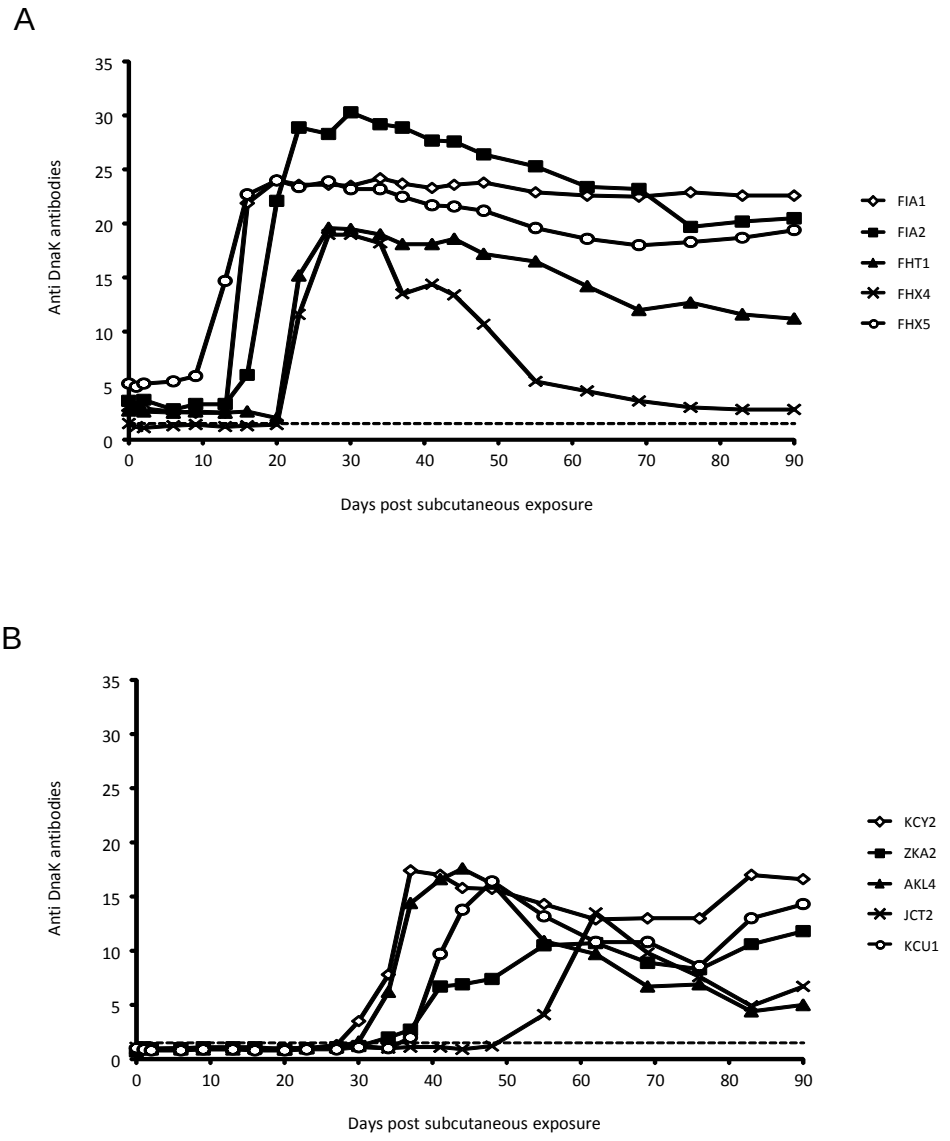


Figure 7: Course of antibody response to Mhf after experimental subcutaneous Mhf exposure (at day 0) in ten SPF cats: five chronically infected cats (A), five naïve cats (B). The antibody levels are presented as ELISA signal-to-noise ratio determined using the Mhf rDnaK ELISA (y-axis). An ELISA signal-to-noise ratio ≥1.5 (indicated by a dotted line) was defined to be positive [18].

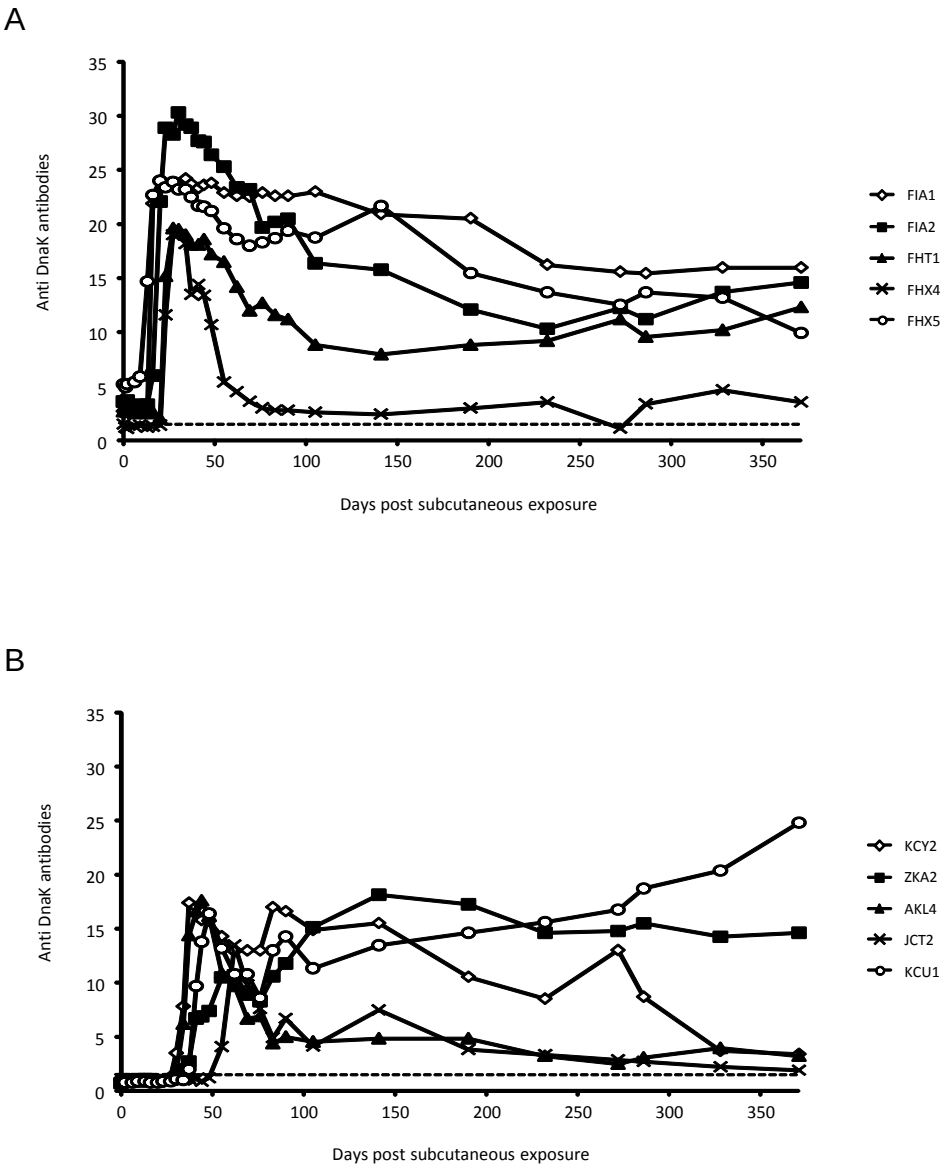


Figure 8: Long-term follow-up of the antibody response to a Mhf infection after experimental subcutaneous Mhf exposure (at day 0) in ten SPF cats: five chronically infected cats (A), five naïve cats (B). The antibody levels are presented as ELISA signal-to-noise ratio determined using the Mhf rDnaK ELISA (y-axis). An ELISA signal-to-noise ratio ≥1.5 was defined to be positive [18].

4. Discussion

This is the first experimental study to show an infection with a low-dose Mhf inoculum and to monitor a possibly cross protection between CMt and the more pathogenic Mhf. In a previous transmission study [17] we demonstrated that subcutaneous inoculation of small volumes of infectious blood is sufficient to transmit a CMt infection and we speculated that this may mimic the natural way of transmission via arthropod vectors or aggressive interaction among cats. Arthropods like fleas or ticks are suspected to play an important role in the transmission of hemoplasmas [11-14]. Hemotropic mycoplasmas have been detected by TaqMan® real-time PCR in ticks and fleas collected from Swiss pet cats [12, 13]. Different studies evaluated risk factors for hemoplasma infection found old male cats with outdoor access more likely to be infected [16, 29]. The increased prevalence was explained by a higher frequency of aggressive contacts in the male cat population and the increased risk of exposure to arthropods in outdoor cats.

We hypothesized that the minimal volume of blood transferred by arthropods or by aggressive contact may also be sufficient to transmit Mhf infection and provoke Mhf bacteremia. Thus, we decided to use a low-dose Mhf inoculum. In all previous experimental studies with Mhf, the intraperitoneal or intravenous inoculation of at least 0.5 mL of Mhf PCR-positive blood was used to induce bacteremia [9, 18-20]. This is the first study demonstrating that a subcutaneous inoculation of 1'000 copies of Mhf corresponding to 0.05 µL of infectious blood with 10^7 copies/mL is sufficient to lead to a Mhf infection. All infected animals developed bacteremia and reached high blood loads (~10^9 copies/mL blood) comparable to reported high-dose experimental Mhf transmission studies [18-20]. However, cats infected subcutaneously with the low-dose inoculum turned PCR-positive later (9 to 34 days post exposure) than what had been

reported for cats inoculated intravenously or intraperitoneally [18, 19]. This fact may depend on the mechanisms by which Mhf reaches the blood stream after subcutaneous inoculation of infectious blood. We suspect that the bacteria were transported by the lymphatic system to finally arrive in the blood vessels. This mechanism is not completely clear, but may be a possible reason for the later appearance of PCR-positive results. Alternatively, the low-dose of the Mhf inoculum may have played a role in the delayed PCR-positivity. After inoculation, the organisms may have been present and even replicating in the blood; however the number of the bacteria may have been below the lower detection limit of the sensitive TaqMan® real-time PCR. Only when the blood loads reached a sufficient concentration (equal to what it would be after intraperitoneal or intravenous inoculation), organisms were detectable by PCR and the kinetics of the infection was similar to what had been reported before for the intraperitoneal or intravenous inoculation. According to our successful low-dose Mhf transmission results, especially veterinarians should be aware of a possible iatrogenic transmission of hemoplasma by minimal volumes of infectious blood, e.g. by multiple uses of needles (vaccination) or surgical materials (castration).

The naïve cats (group B) seroconverted within seven days after the onset of PCR-positivity, a duration that is similar to what had been described in previous studies [18, 23]. Comparing the peak antibody levels between the two groups, significantly higher antibody titers were found in the chronically infected cats compared to the naïve cats. The CMt chronically infected cats showed a boost in antibody levels, three to ten days after turning PCR-positive. The level of antibodies found in the present study for Mhf infection was higher than the level of antibodies reported in CMt infection [24]. This may be due to the higher blood loads reached by Mhf compared to CMt. Furthermore, the higher antibody levels may be explained by the higher affinity of the Mhf antibodies to the antigen, which is based on the sequence of the Mhf DnaK protein [18].

In a previous study, we had found that cats that underwent a CMt infection and recovered from CMt bacteremia subsequently were protected from a second CMt exposure [30]. If these cats were also protected from an infection with the more pathogenic Mhf, there would have been potential for Mhf vaccines based on the less harmful CMt. However, no protection against Mhf was found. In contrast, the chronically CMt-infected cats became PCR-positive significantly earlier than the naïve cats. Remarkably, no CMt was detectable at any time point in any of the cats. Had these cats been naturally infected and diagnostic PCR been run for Mhf and CMt, no difference would have been notable between cats in groups A and B (all just positive for Mhf), although they clearly show different infection kinetics. Thus, previous hemoplasma infections from which the cats have recovered may influence subsequent hemoplasma infections; this observation may contribute to the fact that natural hemoplasma infections may demonstrate differently in individual cats.

There are several possible explanations for the unexpected finding that chronically CMt-infected cats became significantly earlier PCR-positive than naïve cats. Pre-existing antibodies against hemoplasmas may have had an impact on the kinetic of the Mhf infection in cats that recovered from CMt infection. The reason behind this mechanism, the so-called antibody-dependent enhancement (ADE), is that the presence of specific antibodies against an infectious agent, can lead to an increased replication and distribution of the pathogen. ADE is particularly known in virus infections, e.g. feline infectious peritonitis virus, dengue virus, feline or human immunodeficiency virus [31-34], but it has also been described in bacteria. The specific antibody response against *Streptococcus pneumonia* can enhance the adherence to and the colonization of respiratory cells [35, 36]. In our study, the chronically infected cats have antibodies against hemoplasmas prior to the Mhf exposure, as opposed to the naïve cats in group B. These antibodies may have bound to Mhf and the antibody-hemoplasma complex

may have been incorporated by phagocytic defense cells. This is based on the assumption that Mhf would be able to replicate and spread on a higher number from these cells. However, the replication of Mhf in/on cells other than erythrocytes has not been demonstrated [37]. For CMt and CMt reinfection no ADE has been found [30]. Alternatively, a modulation of the immune system may be responsible for the fact that chronically CMt infected cats turned Mhf PCR-positive earlier than the naïve cats. The influence of a hemotropic mycoplasma infection to the host immune system has not yet been completely elucidated. The humoral and the cellular immune system seem to play a role in the control of a hemoplasma infections [23, 24, 30, 38]. In a non-hemotropic mycoplasma infection, it has been reported that calves infected with *Mycoplasma bovis* showed a down-regulation of the immune system [39]. If chronically CMt-infected cats also undergo a down-regulation of the immune system, this may have led to a faster replication of Mhf and earlier PCR-positivity in the blood of cats in group A. Moreover, a modification or upregulation of potential hemoplasma binding receptors on the erythrocyte surface may also play a role in the binding of hemoplasma to the red blood cells. There has been evidence that patients with acute, severe hemoplasma infection had significantly elevated levels of complement receptors (CD35) compared to healthy controls [40]. Assuming that chronically infected cats had a similar up-regulation of potential hemoplasma receptors, they may have allowed for a more efficient binding of Mhf to the red blood cells and thus a faster replication. However, again, this was not found for CMt reinfection in chronically CMt-infected cats [30].

All cats reached high blood loads (~10^9 copies/mL), although the cats were infected with a low-dose Mhf inoculum. Apart from one cat, the high Mhf blood loads were associated by a marked decrease in PCV. While several cats showed only mild clinical signs of hemoplasmosis (depression, loss of appetite, pale mucous membranes), one cat (cat JCT2) developed severe anemia (PCV = 13%), which necessitated antibiotic treatment.

Thus, apart from the Mhf isolate, the inoculation route, dose and previously undergone hemoplasma infections, apparently additional individual factors may play a role in the susceptibility of different cats, e.g. the immune status or genetic background of the cat. An influence of the blood type, as previously postulated [17], can be excluded in the present study, because all cats were of blood type A.

Some marked copy number fluctuations were observed during the course of Mhf infection also after the low-dose exposure – as has been reported after natural infection and high-dose infection [16, 18]. The fluctuation was especially pronounced in the early phase and in one cat in group B (ZKA2). This cat showed characteristic blood load cycling with three distinct Mhf load peaks between 23 and 48 days post infection. The fluctuations ranged from 10^4 to 10^8 copies/mL blood within a minimum of three days.

Moreover, in accordance with natural and experimental high dose Mhf infections, Mhf was also detectable by light microscopy in the modified Wright stained blood smear prepared with blood collected during bacteremia. Mhf appeared as small coccoid structures on the surface of the red blood cells, as described [22]. Furthermore, anisocytosis, polychromatic erythrocytes and normoblasts were seen compatible with regenerative anemia. One month after the cats turned PCR-positive, a decrease in eosinophiles and simultaneously an increase in monocytes was observed. These changes are often associated with acute infections [41, 42].

Several changes in clinical chemistry were reported for the first time in feline hemoplasma infections. A significant increase in total protein concentration was observed one month after the cats had become PCR-positive. The increase in total protein was paralleled by a decrease in albumin, which may be explained by the role of albumin as negative acute phase protein [43]. Different factors like dehydration, liver and kidney disease have an influence on the level of proteins [43]. In the present study, the increase in total protein concentration during hemoplasma infection may be mainly

attributed to an increased production of immunoglobulins. This assumption was supported by the hypergammaglobulinemia detected in the serum protein electrophoresis in nine out of ten cats. Furthermore a decrease in alkaline phosphatase and cholesterol was seen during the observation time. Considering that the half-life of alkaline phosphatase in cats is very short, it is difficult to interpret these values. The Mhf infection may lead to a general increase of the metabolic activity, which may result in an additional decrease of the half-life of alkaline phosphatase. Cholesterol plays an important role in the construction of cell membranes and the production of hormones and bile acid. Alterations in the lipid metabolism and decreased cholesterol levels have been reported in a many infections, including viral, bacterial and parasitic infections [44, 45]. The observed decrease in cholesterol concentration in the present study may be due to an increased need of cholesterol for membrane synthesis for either large numbers of RBCs that have been destroyed during the Mhf infection and/or – what seems more probable – the fast amplification of hemoplasmas. It has been shown that mycoplasmas depend on the host to provide cholesterol for the synthesis of their membranes [39]. Due to their small genome, they have limited metabolic options for survival and replication and depend on exogenous substrates [46, 47]. In addition, they incorporate large quantities of cholesterol into their membrane for improved membrane fluidity [39]. High levels of cholesterol have been shown to enhance replication of some bacteria and cholesterol catabolism may be a therapeutic target against these infections [45]. Moreover, a significant increase in bilirubin concentration was observed. As described earlier [10], an acute Mhf infection may lead to hemolytic anemia, which may result in a rise of bilirubin concentration. In addition, cat JCT2 showed an increased ASAT (108 U/l) and ALAT (806 U/l) at day 69 post infection; these elevations could be explained by the foregoing treatment with doxycycline. Cats in group B showed similar tendencies in clinical chemistry, but as a group not as pronounced as cats in group A.

This may be due to fact that the time points of PCR conversion in the naïve cats was spread more widely over time compared to the chronically infected cats. The changes observed in clinical chemistry and the serum protein electrophoresis were not high enough to potentially aid establish an accurate diagnosis but they add new insights into the infection pathogenesis.

Finally, the shedding patterns of Mhf were investigated. The present study reports for the first time, that Mhf DNA was shed by saliva, feces and urine. Positive saliva and rectal swabs could be found particularly during high bacteremia. However, all Mhf-positive swabs showed a low hemoplasma load, comparable to the loads demonstrated for CMt [13, 17]. The saliva and rectal swabs collected at later time points, > 100 days post inoculation, were all negative. So far there have been no Mhf transmission studies using PCR-positive saliva or feces. For CMt, it has been demonstrated that PCR-positive saliva does not pose a significant risk of infection transmission, when inoculated either orally or subcutaneously [17]. In addition, Mhf PCR-positive urine samples were found in the present study. The urine samples were collected by cystocentesis; therefore a contamination with erythrocytes cannot be completely excluded. However, Mhf PCR-positive results were also detected in urine samples, where no erythrocytes were detected by urinalysis and in the urine sediment. The low Mhf copy numbers in saliva, feces and urine of Mhf-infected cats are indicative for a low risk of transmission by sharing of feeding dishes, toys or cat toilets by Mhf PCR-positive and -negative cats.

5. Conclusion

In conclusion, we demonstrated that cats that had recovered from CMt infection were not protected against a subsequent Mhf challenge. In contrast, they became PCR-positive and anemic significantly earlier than the naïve cats. Our study demonstrated that a minimal contact to Mhf infectious blood was sufficient for the transmission of the infection and induction of hemoplasmosis. Thus, we propose to use the low-dose challenge in future hemoplasma studies to reflect the natural way of transmission. Moreover, we demonstrated for the first time that Mhf is shed by saliva, feces and urine especially during the time of high bacteremia. In addition, it was found that approximately half of the Mhf-infected cats may become PCR-negative even in the absence of antibiotic treatment as had been reported for most CMt-infected cats, while others stayed Mhf PCR-positive in the blood for more than a year after Mhf infection, similar to what was mostly seen for CMhm.

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